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A treatment that eliminates SARS-CoV-2 replication in human airway epithelial cells and is safe for inhalation as an aerosol in healthy human subjects. Michael D. Davis, RRT, PhD, FAARC^{1, 2}, Tatiana M. Clemente, PhD³, Olivia K. Giddings, MD, PhD⁴, Kristie Ross, MD⁴, Rebekah S. Cunningham¹, Laura Smith¹, Edward Simpson^{5, 6}, Yunlong Liu^{5, 7}, Kirsten Kloepper, MD², I. Scott Ramsey, PhD⁸, Yi Zhao, PhD⁹, Christopher M. Robinson, PhD³, Stacey D. Gilk, PhD³, Benjamin Gaston MD^{1, 2}

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Author contributions

MDD performed experiments and helped prepare the manuscript. TMC aided in the design and execution of viral experiments, as well as data analysis. OKG conducted the *in vivo* studies and aided in drafting of figures in this manuscript. KR is PI of the *in vivo* studies and provided oversight of work at Case Western Reserve University. RSC conducted airway epithelial cell experiments and pH assays. LS oversaw all airway epithelial cell experiments and aided in drafting methods and figures. ES and YL performed sequencing analysis and aided in drafting methods and figures. KK is PI of the pediatric sample biobank at Indiana University and aided with study design. ISR designed airway epithelial pH experiments and aided with drafting the manuscript. YZ was the primary biostatistician for this project and aided with methods and figures. CMR designed viral experiments, optimized viral expansion/inoculation methods, and aided with figures. SDG is PI for the BSL-3 facility, designed and provided oversight for all viral experiments, and assisted with methods. BG was PI, conceived of the project, oversaw the work and prepared the manuscript.

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Competing Interests

MDD and BG are funded by NIH P01 HL128192-01A1, are patent holders of Optate, and are co-Founders of Airbase Breathing Company.

SDG and CMR are also patent holders of Optate.

Abstract

Background: Low airway surface pH is associated with many airway diseases, impairs antimicrobial host defense and worsens airway inflammation. Inhaled Optate is designed to safely raise airway surface pH and is well-tolerated in humans. Raising intracellular pH partially prevents activation of SARS-CoV-2 in primary normal human airway epithelial (NHAE) cells, decreasing viral replication by several mechanisms.

Methods: Here, we grew primary normal human airway epithelial (NHAE) cells from healthy subjects, infected them with SARS-CoV-2 (isolate USA-WA1/2020), and used clinical Optate at concentrations used in humans *in vivo* to determine whether it would prevent viral infection and replication. Cells were pre-treated with Optate or placebo prior to infection (MOI of 0.1) and viral replication was determined by plaque assay and nucleocapsid (N) protein levels. Healthy human subjects also inhaled Optate as part of a Phase 2a safety trial. **Results:** Optate almost completely prevented viral replication at each time point between 24 and 120 hours, relative to placebo, both by plaque assay and by N protein expression ($p < 0.001$). Mechanistically, Optate inhibited expression of major endosomal trafficking genes and raised NHAE intracellular pH. Optate had no effect on NHAE cell viability at any time point. Inhaled Optate was well tolerated in 10 normal subjects, with no change in lung function, vital signs or oxygenation.

Conclusions: Inhaled Optate may be well-suited for a clinical trial in patients with a pulmonary SARS-CoV-2 infection. However, it is vitally important for patient safety that formulations designed for inhalation with regards to pH, isotonicity and osmolality be used. An inhalational treatment that safely prevents SARS-CoV-2 viral replication could be helpful for treating patients with pulmonary SARS-CoV-2 infection.

Key Words

Airway pH, COVID-19, SARS-CoV-2.

Introduction

Airway surface lining fluid (ALF) coats the extracellular space of the epithelial cells that line the airways. ALF acidification contributes to the pathophysiology of asthma and other respiratory diseases¹⁻³. Specifically, ALF acidification is known to increase cough and bronchospasm, facilitate bacterial growth, increase mucus viscosity, decrease ciliary beat frequency, and cause general epithelial dysfunction^{2, 4-10}. ALF acidification occurs during inflammatory and infectious airway disease exacerbations^{2, 6, 8-12}. Therefore, we have been investigating the effects of raising human ALF pH using an inhalational treatment, Optate (Airbase Breathing Company, Indianapolis, IN). Optate is an inhaled isotonic, isosmotic, alkaline medication designed to safely modify airway pH without irritating the airway epithelium. We have previously demonstrated in several safety trials (Phase 1 and 2) that Optate inhalation is well tolerated in healthy humans and those with stable asthma and COPD⁹. We also demonstrated that a single inhalation of Optate effectively raises ALF pH, indicated by a decrease in exhaled nitric oxide and an increase in exhaled breath condensate pH⁹.

Pandemic infection with SARS-CoV-2 (COVID-19) has cost hundreds of thousands of lives within the last year (www.who.int, accessed 08/09/2020). *In vitro*, raising intracellular pH partially prevents activation of SARS-CoV-2 in primary normal human airway epithelial (NHAE) cells; indeed, intracellular alkalinization decreases viral replication by several mechanisms¹³⁻¹⁶. Because Optate is effective at raising

extracellular pH in the human airway, we studied whether it would prevent SARS-CoV-2 replication in NHAEC cells. Specifically, we hypothesized that Optate would increase intracellular pH and decrease SARS-CoV-2 replication in NHAEC cells compared to those treated with a placebo with the hope that it could serve as a treatment for this disease.

Methods

Cell culture. Primary NHAEC cells were grown as previously described from healthy, non-smoking donors^{17, 18}.

Study treatments. 120 mM Optate was prepared and assayed for purity, potency, osmolality (target ~330 mOsm), pH (target 9.8) and sterility prior to all experiments (IND #139144; Arena District Pharmacy, Columbus, Ohio, USA). For the *in vivo* study, 10 healthy subjects > 18 y/o with no history of lung disease was recruited under our IRB-approved protocol (Case Western Reserve University IRB #03-18-28). Forced expiratory volume in one second (FEV₁), forced vital capacity (FVC), heart rate (HR), oxygen saturation (SpO₂) and adverse events were monitored before and after one 2.5 mL nebulization of 120 mM Optate as previously described⁹ and reviewed by our Data Safety Monitoring Board. No change was noted in any vital signs of any subject before and after treatment.

For the *in vitro* studies, cells were exposed to the same batch of 120 mM Optate described above 1:1 with cell culture media to mimic the expected dilution effects of airway lining fluid *in vivo*. Phosphate-buffered saline (PBS) was used as the *in vitro*

placebo. Airway cells were acquired by brush biopsy with informed consent under Indiana University Institutional Review Board Protocol #1408855616.

Intracellular pH assays. Intracellular pH was evaluated with fluorescent dyes: 1.25 μ M 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester (BCECF-AM, Invitrogen™, ThermoFisher Scientific, USA), which increases in green fluorescent intensity (E:E 500:531 nm) as pH increases¹⁹; and 20 μ M pHrodo Red (PR, Invitrogen™, ThermoFisher Scientific, USA), which decreases in red fluorescent intensity (E:E 560:585 nm) as pH increases²⁰. After washing (PBS) cells were treated with Optate (clinical solution, 1:1 dilution in medium) or PBS. As a negative control, cells were treated with ZnCl₂, (100 μ M) which acidifies the intracellular space by inhibiting Hv1²¹.

Viral growth and plaque assays. SARS-CoV-2 Isolate USA-WA1/2020 was provided by Biodefense and Emerging Infection (BEI) Resources (Manassas, VA). In our BSL3 facility, viral stocks were prepared in Vero E6 cells (African green monkey kidney cell line, ATCC™, Manassas, VA) at 37°C for 2-4 days until cytopathic effect (CPE) was observed. Media from the cells were collected and centrifuged (1000xg; 5 min). Virus was quantified by plaque assay (See details in the Supplemental methods).

Vero E6 or NHAE cells were plated the day prior to the experiment. Cells were pre-treated with Optate or PBS for 5-10 minutes prior to SARS-CoV-2 infection (MOI of 0.1). After one hour, cells were washed with PBS, and medium with or without Optate was added. For viral titers, the media was not changed prior to harvesting supernatant for viral titers at 24 hours post infection. Supernatant was harvested every 24 hours and viral replication determined by plaque assay. Data are from three experiments, each

done in duplicate. NHAIE infection studies used two different donors to reduce donor effect on results.

Immunoblots. Using the same protocol as that for plaque assay (above) cells were lysed in RIPA buffer. Capillary electrophoresis was performed on the automated JESS system (ProteinSimple™, San Jose, CA). Briefly, 0.5ug/ul lysate was plated and run following manufactures recommendations. Antibodies are listed in the supplemental methods. Compass software (ProteinSimple™) generated digitally-rendered bands based on chemiluminescence electrophoretogram.

RNA processing. RNA was extracted from control and Optate-treated NHAIE cells (48 hr) using RNeasy™ Plus kit (Qiagen, Hilden, Germany) following the manufacturer-recommended protocol. Total RNA was first evaluated for its quantity, and quality (Agilent Bioanalyzer 2100); all Samples had RNA Integrity Number of 9 or higher. Total RNA (100 ng) was used for cDNA library preparation and quantitation, as described in the supplemental methods. More than 95% of the sequencing reads reached Q30 (99.9% base call accuracy).

Sequenced libraries were mapped to the human genome (UCSC hg38) using STAR RNA-seq aligner (v.2.5) with the following parameter: "--outSAMmapqUnique 60." Read distribution across the genome was assessed using bamutils (from ngsutils v.0.5.9). Uniquely mapped sequencing reads were assigned to hg38 refGene genes using featureCounts (subread v.1.5.1) with the following parameters: "-s 2 -Q 10". Reads with count per million < 0.5 in more than three samples were removed.

Cytotoxicity assays. Cytotoxicity of Optate was measured both by lactate dehydrogenase (LDH) concentration (Cayman Chemical assay) and by Trypan Blue exclusion (Bio-Rad TC20™ automated cell counter) according to manufacturer specifications as previously described²².

Data analysis. Student's unpaired, two-tailed t-test was used to evaluate cytotoxicity, pH, viral plaque, and protein levels comparing control groups and treatment groups. Wilcoxon rank-sum test was used for non-Gaussian distributed data as determined by the Shapiro-Wilk test. Mixed effects ANOVA model was used for repeated measures, with a Tukey's post-hoc correction for multiple comparisons. For RNA sequencing, gene symbols were converted to entrez ids using *biomaRt* and analyzed with the *clusterProfiler* package in R^{23, 24}. Data were normalized using trimmed mean of M values. Differential expression analysis was performed using edgeR (v.3.12.1). Family error rate was controlled for using Bonferroni.

Results

Optate raises intracellular pH in NHAEC cells. After loading with BCECF-AM (green) or PR (red) dye, cells were treated with Optate or placebo and imaged for intracellular fluorescent intensity. Cells treated with Optate showed a significant increase in green fluorescent intensity (n = 4, p = 0.039) and decrease in red fluorescent intensity (n = 4, p = 0.001) compared to those treated with placebo, indicating an increase in intracellular pH (Figure 1, A and B). ZnCl₂, which is known to acidify the intracellular space²¹, had an effect opposite Optate (n = 3 and 5, p = 0.036 and 0.001, Figure 1B).

Optate decreases SARS-CoV-2 viral replication in Vero E6 and NHAE cells. SARS-CoV-2 plaque forming units (PFUs) were measured in culture media from Optate- and placebo-treated Vero E6 cells (the cells used to expand viral stock) infected with SARS-CoV-2. All cells were treated one-time, immediately after infection. Optate ablated viral PFUs in the Vero E6 cells ($n = 3$ experiments, two replicates each, $p < 0.001$, Figure 2A). Under similar conditions, NHAE cells were then infected with SARS-CoV-2, treated with Optate or placebo, and SARS-CoV-2 PFUs and N protein levels were measured daily for five days. After infection was established (24 hours), both viral PFUs and N protein levels were ablated in the Optate-treated cells unlike the placebo-treated cells (3 experiments using cells from two subjects, two replicates each, $p < 0.001$, Figure 2B-D).

Optate alters endocytosis and endosomal trafficking genes in NHAE cells. RNA sequencing results were compared between control and Optate-treated NHAE cells. Genes with significant differences between the groups are presented in a volcano plot (Figure 3). Genes decreased in the Optate-treated group are negative (left) and those that increase are positive (right). Downregulated genes for endocytosis and endosomal trafficking are labeled in green. Genes in blue have a Bonferroni-corrected p -value < 0.001 and the absolute $\log_{(FC)} < 1.5$; magenta are Bonferroni corrected p -value < 0.001 and the absolute $\log_{(FC)} > 1.5$. Genes in black are below the significance threshold of 0.001.

Optate is safe and well tolerated in healthy human subjects *in vivo* and *in vitro*. Human subjects who inhaled 120 mM Optate had no significant change in FEV1 ($p = 0.84$), FVC ($p = 0.99$), HR ($p = 0.42$), or SpO₂ ($p = 0.99$) after inhalation, consistent with previous data (Figure 4A, $n = 10$ normal volunteers)⁹. To validate that the observed

antiviral effects were not due to Optate killing the host cells, we evaluated cytotoxicity in the control and Optate-treated cells. Primary normal human airway epithelial (NHAE) cells exposed to 120 mM Optate, did not differ from the control group in cytotoxicity evaluated by Trypan Blue exclusion or lactate dehydrogenase (LDH) levels (Figure 4B).

Discussion

Our group has previously shown that inhaled compounds can alter airway pH *in vivo*^{1, 9}. Alterations of airway pH for therapeutic purposes have been proposed for chlorine gas inhalation, asthma, and cystic fibrosis^{1, 25, 26}. These applications have all focused on altering extracellular pH, likely due to the fact that most commonly known toxicities of ALF pH disturbance are extracellular. However, most respiratory viruses require an intracellular acidic event within airway epithelial cells in order to enter and/or replicate^{13, 16}. Our findings above illustrate that Optate, which has been previously shown to increase ALF (extracellular) pH⁹, indeed raises the intracellular pH of NHAE cells and ablates SARS-CoV-2 viral replication post-infection. A safe, inhaled medication for raising intracellular pH could be of benefit for treating respiratory viral diseases even beyond SARS-CoV-2.

This work has several limitations. As with any *in vitro* experiment, the cell culture models used here are not necessarily representative of the *in vivo* systems and disease processes and our observations may translate. Moreover, COVID-19 involves many physiologic processes downstream from SARS-CoV-2 infection and replication. It is possible that different therapeutic targets rather than viral replication will be necessary to benefit patients. However, the fact that Optate inhalation does alkalinize the extracellular milieu *in vivo* does suggest the observed effects may occur in patients with

SARS-CoV-2. Thus, we are preparing to conduct a randomized, placebo-controlled, multicenter trial to better evaluate the therapeutic value of Optate. There were also technical limitations regarding the experiments used. For instance, viral infection of NHAEC cells took over 24 hours and resulted in lower titers than in Vero E6 cells. This was previously observed in NHAEC infection models of SARS-CoV-1^{27, 28} and therefore unsurprising, however, it did warrant validation in several replicates and the use of multiple cell lines. Further studies are warranted to optimize this model, including evaluation of different time courses, the ability for viral replication to occur after treatment cessation, and the duration of effects. Finally, although our results suggest our mechanism of action results from intracellular alkalinization leading to altered endocytic pathways and endosomal trafficking, there could be other mechanisms by which Optate ablates SARS-CoV-2 infection in Vero E6 and NHAEC cells.

Inhalation of pH-altering compounds should be done with caution. Exposures to pH above 10 and below 6.5 are known to adversely effect NHAEC cell function^{7, 22}. Moreover, inhalation of hypotonic and hyposmotic solutions, such as many intravenous medications, causes airway irritation, bronchial hyperreactivity and coughing²⁹. Our 1:1 PBS/cell culture media placebo group had a pH of 8 and still had significant viral infection, suggesting a fairly narrow therapeutic window for this purpose. Therefore, a specific formulation designed for inhalation with regards to isotonicity and osmolality that is buffered to an ideal pH within the therapeutic window needed to inhibit viral replication is required.

Conclusion

Optate raises intracellular pH and prevents SARS-CoV-2 replication in NHAEC cells.

Inhaled Optate raises ALF pH in human subjects. It is non-toxic *in vitro* and *in vivo*, suggesting that a clinical trial to prevent treat life-threatening SARS-CoV-2 respiratory infection could be contemplated.

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Quick Look

Current Knowledge. Low airway epithelial surface pH impairs antimicrobial host defense and worsens airway inflammation. Low airway epithelial intracellular pH

facilitates SARS-CoV-2 cell entry and replication. Inhaled Optate is designed to raise airway epithelial surface pH.

What This Paper Contributes to Our Knowledge. At the dose inhaled in human trials, Optate raises airway epithelial intracellular pH and almost completely prevents SARS-CoV-2 replication in NHAE cells, ablating both viral plaque formation and nucleocapsid protein expression. Optate is thus an effective anti-viral *in vitro* that is established to be safe for human inhalation in healthy subjects.

Figure Legends

Figure 1. Optate raises intracellular pH of NHAE cells. (A) Optate raised intracellular pH. NHAE cells were loaded with BCECF-AM, which increases in fluorescence intensity as pH increases across the physiological range¹⁹ (top), or pHrodo Red, which decreases in fluorescence intensity as pH increases²⁰ (bottom). Cells were imaged before or after treatment. (B) Fluorescent intensity increased in cells loaded with BCECF-AM and decreased in cells loaded with PR (n = 4 each; p = 0.039 and 0.001, respectively). As a control, we treated cells with ZnCl₂, which acidifies the intracellular space by inhibiting voltage-gated proton channel Hv1²¹. Zinc had an effect opposite Optate (n = 3 and 5; p = 0.036 and 0.001, respectively). Means are reported with wide bars and standard error is indicated by whiskers.

Figure 2. Optate decreases SARS-CoV-2 viral replication in Vero E6 and NHAE cells. (A) PFUs were measured in culture media from control and Optate-treated Vero E6 cells infected with SARS-CoV-2. Optate ablated viral infection in the Vero E6 cells (n = 3 experiments, two replicates each, p < 0.001). Means are represented with wide bars

and standard error is represented by whiskers. **(B)** Control and Optate-treated primary NHAIE cell cultures were then infected with SARS-CoV-2 and PFUs in culture media were analyzed under similar conditions from 24 hr for 120 hr after infection. After infection was established (24 hours), viral infection was ablated in the Optate-treated cells (3 experiments using cells from two subjects, two replicates each, $p < 0.001$). Dots represent means and whiskers represent standard error. **(C and D)** N protein expression was then studied in cell lysates from NHBE cells described in 2B. SARS-CoV-2 N protein expression, normalized to β -actin, was almost completely suppressed at time points after 24 hours ($p < 0.001$). Means are represented with wide bars and standard error is represented by whiskers.

Figure 3. Optate alters endocytosis and endosomal trafficking genes in NHAIE cells. RNA sequencing results were compared between control and Optate-treated NHAIE cells. Genes with significant differences between the groups are presented in a volcano plot. Genes decreased in the Optate-treated group are negative (left) and those that increase are positive (right). Downregulated genes for endocytosis and endosomal trafficking are labeled in green. Genes in blue have a Bonferroni-corrected p -value < 0.001 and the absolute $\log_{(FC)} < 1.5$; magenta are Bonferroni corrected p -value < 0.001 and the absolute $\log_{(FC)} > 1.5$. Genes in black are below the significance threshold of 0.001. A list of downregulated genes is provided in the supplemental results.

Figure 4. Optate safely is well tolerated in the human airway *in vitro* and *in vivo*.

(A) Human subjects had no significant change in FEV1 ($p = 0.84$), FVC ($p = 0.99$), HR ($p = 0.42$), or SpO_2 ($p = 0.99$) after Optate inhalation ($n = 10$ normal volunteers). Dots

represent means. **(B)** NHAEC cells exposed to Optate for five days did not differ from the control group in cytotoxicity evaluated by LDH levels (Top) or % viability determined by Trypan Blue exclusion (Bottom). Means are represented with wide bars and standard error is represented by whiskers.

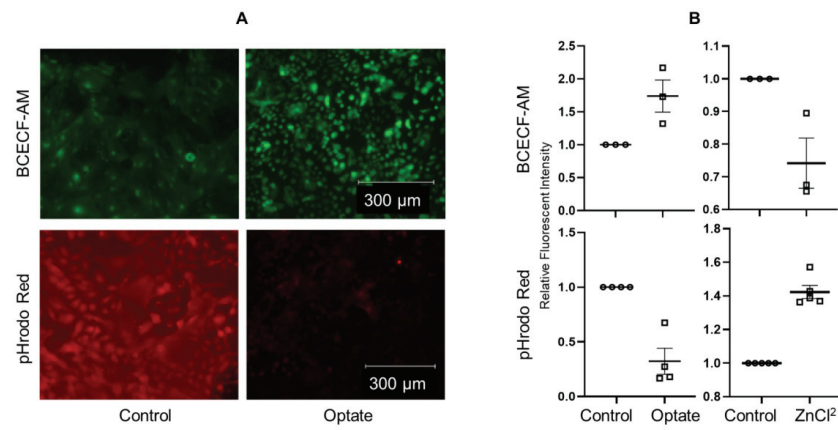


Figure 1

Figure 1

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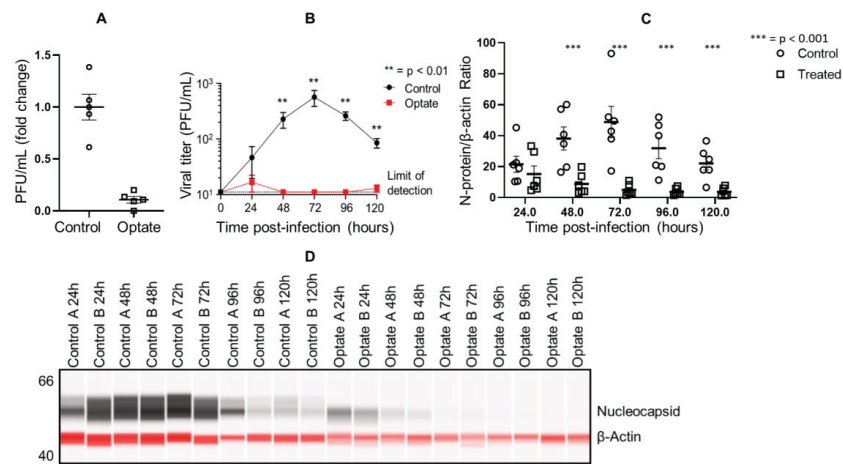


Figure 2

Figure 2

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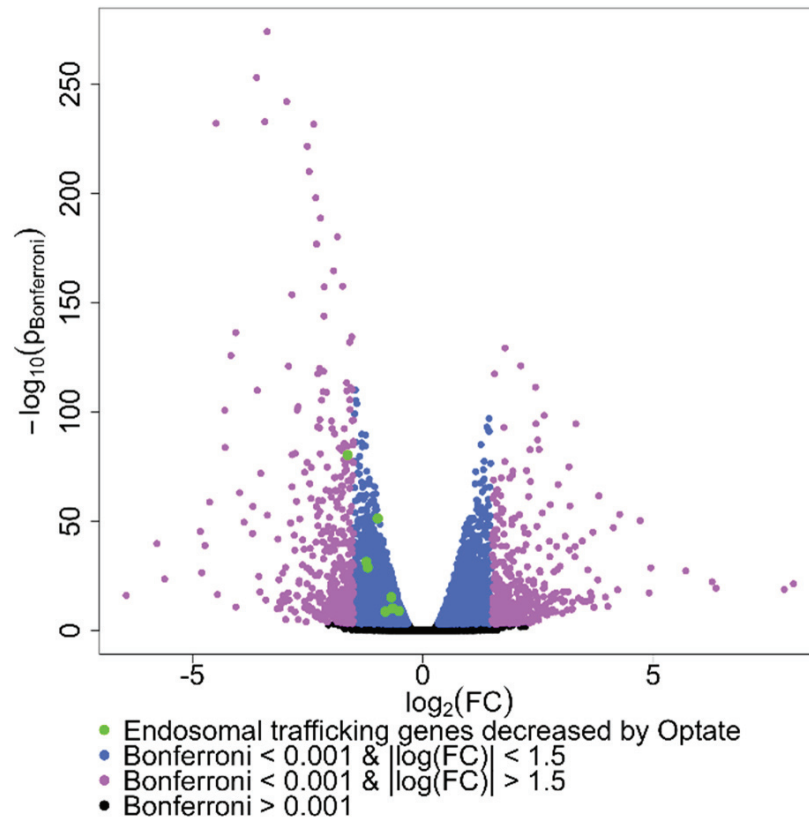


Figure 3

Figure 3

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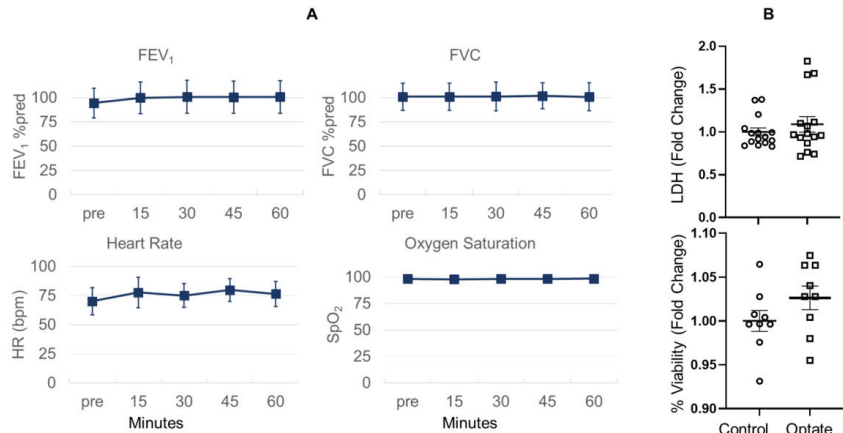


Figure 4

Figure 4

292x170mm (150 x 150 DPI)